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Solution *versus* Solid-phase Cyclization Strategies for Large Sidechain Lactam-bridged Peptides: A Comparative Study

JULIO A. CAMARERO, JORDI J. CAIRÓ, ERNEST GIRALT and DAVID ANDREU

Department of Organic Chemistry, University of Barcelona, Barcelona, Spain

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Abstract: A 22-residue peptide with a sidechain lactam bridge involving 18 residues (60-atom cycle) has been synthesized. Three different protection schemes using Fmoc/^{t}Bu/cyclohexyl, Fmoc/^{t}Bu/allyl or Boc/Bzl/fluorenylmethyl protecting group combinations have been explored for the solid phase of the linear precursors, which have been subsequently cyclized in solution or in the solid phase. Cyclization yields in solution have been consistently better than on solid phase; however, the solid-phase strategy requires fewer purification steps and therefore global yields are comparable.

Keywords: Cyclic peptides; large ring size peptides; sidechain lactam formation; solid-phase peptide cyclization; foot-and-mouth disease virus peptides

Abbreviations

AAA, amino acid analysis; All, allyl; Aloc, allyloxycarbonyl; AM, 2-[4-(9-fluorenylmethoxycarbonyl)-aminomethyl(2,4-dimethoxyphenyl]-phenoxy)acetic acid; BOP, benzotriazole-1-yl-oxy-*tris*(dimethylamino)phosphonium hexafluorophosphate; ^{t}Bu, *tert*-butyl; Bzl, benzyl; cHex, cyclohexyl; DBU, 1,8-diazabicyclo[5.4.0]undec-7-ene; DCM, dichloromethane; DIEA, *N,N'*-diisopropylethylamine; EDT, 1,2-ethanedithiol; ES-MS, electrospray mass spectrometry; FAB-MS, fast atom bombardment mass spectrometry; Fm, 9-fluorenyl; Fmoc, 9-fluorenylmethyloxycarbonyl; HPCE, high-performance capillary electrophoresis; HPLC, high-performance liquid chromatography; IRA, internal reference amino acid; *p*-MBHA, *p*-methylbenzhydrylamine; MPLC, medium pressure liquid chromatography; PEG–PS polyethylene glycol–polystyrene resin; Pmc, 2,2,5,7,8-pentamethylchroman-6-sulfonyl; PyBOP, benzotriazole-1-yl-oxy-tris(pyrrolidino)phosphonium hexafluorophosphate; TBTU, 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate; TFA, trifluoroacetic acid; THF, tetrahydrofuran; Tos, *p*-toluenesulfonyl; UV, ultraviolet.

The possibility of inducing preferred bioactive conformations in linear peptides through restricted mobility congeners is one of the main reasons behind the growing interest in cyclic peptides [1–3]. The limited flexibility of the cyclic versus the linear structures often results in enhanced binding and improved pharmacological properties.

Classical methods of cyclic peptide synthesis [4] generally rely on fully protected linear precursors which are selectively activated and cyclized under highly dilute conditions. This approach often suffers from the insolubility of protected peptides as their size increases. In recent years many efforts have been devoted to the synthesis of cyclic peptides in the solid phase [5]. This approach benefits from the inherent advantages of the solid-phase method [6], particularly the easy removal of excess reagents from the resin-bound peptide, simplifying the isolation and purification steps. The pseudo-dilution conditions [7, 8] required to avoid competing inter-site reactions

Address for correspondence Dr David Andreu; Department of Organic Chemistry, University of Barcelona, Martí i Franquès 1; E-08028 Barcelona, Spain

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leading to oligomers can usually be achieved by adequate choice of low-substitution resins. On the other hand, an obvious requirement for successful on-resin cyclization is a highly efficient synthesis of the linear (on-resin) precursor since, in contrast to conventional methods, no possibility of purification of the starting material exists.

Most efforts in the area of on-resin cyclizations have concentrated on peptides of 'ideal' cycle size, usually in the 4–12 residue range, via either amide bonds [9–15] or disulfide bridges [16–20]. In contrast, there are few examples of large, complex (*e.g.* with a number of other trifunctional residues) cyclic peptides made on solid support [21]. We report the synthesis of a large peptide of this type via sidechain-to-sidechain amide bond formation, and compare the relative merits of the solution and solid-phase cyclization strategies. For this purpose we have chosen as the synthetic target the 22-residue peptide **1** (Figure 1), a His-rich sequence that mimics the G–H loop of foot-and-mouth-disease virus protein VP1 [22] and is useful in immunological studies.

In designing strategies for the synthesis of **1**, three alternative protection schemes were explored for the solid-phase synthesis of the linear precursors (Figure 2, where R and R′ symbolize *p*-MBHA and AM–IRA–PEG–PS [23] resins respectively), which were cyclized in solution (strategy A) or on the resin (strategies B and C). All three schemes have been devised to allow regiospecific sidechain-to-sidechain amide bond formation between residues Lys^{20} and Glu^{3}, which in turn depends on orthogonal protection for either these two residues (strategies B and C) or for the carboxyl sidechain of Asp^{10} and the N-terminal amino group (strategy A).

Strategy A

The solid-phase synthesis of the linear precursor was based on Fmoc/^{t}Bu chemistry [24]. The protection scheme was designed to give a linear precursor in which the Asp^{10} carboxyl and N-terminal amino groups, not being involved in sidechain lactam cyclization, were protected. For this purpose, the cyclohexyl (OcHex) and benzyloxycarbonyl (Z) groups, both stable to TFA, were chosen, respectively. The remaining sidechains were protected with TFA-labile groups that would produce a partially deprotected linear precursor, with free hydroxyl, imidazole and guanidino groups. In choosing this approach, the predictable improved solubility and resulting ease of purification of the linear precursor was considered more important than the potential risk of side reactions from these functions. The alcohol groups of Thr and Ser residues have been shown to be very unreactive towards esterification when phosphonium salts are used as amide bond formation reagents [25]. Threonine, in addition, is very sterically hindered. In the Arg residue, the guanidinium function is protonated after the cleavage with TFA and is thus essentially trouble-free.

The protected peptide resin was submitted to TFA acidolysis and the resulting material was purified by preparative MPLC to give partially protected [Z-N^{α},Asp^{10}(OcHex)] linear peptide **1**, which was correctly characterized by amino acid analysis and FAB-MS. It is of interest to note that when the TFA cleavage was carried out in the presence of thioanisole (as in reagent R [26]), significant loss of the Z-N^{α} protection was observed (Figure 3A, trace 2). This was minimized with other cleavage reagents such as reagent B [27] or TFA/H_2O/anisole. Cyclization was accomplished under high-dilution conditions using a large excess of either BOP or PyBOP as coupling agent (20 equiv. added every 6 h) in DMF containing 0.5% (v/v) DIEA for 18 h at room temperature. HPLC profiles of the cyclization crudes (Figure 3B) showed a major component regardless of the coupling reagent used, although slightly better results were accomplished with BOP. Without further purification, the crude cyclic peptide was submitted to HF acidolysis (Figure 3C) to remove its remaining protecting groups, then purified by semipreparative HPLC to yield the desired product (Figure 3D). Cyclic lactam **1** was correctly characterized by ESMS and tryptic digestion, the latter method allowing unequivocal confirmation of the correct connectivity of the new sidechain-to-sidechain bond.

Strategy B

This approach is quite similar to one previously used [14, 15] in the synthesis of cyclic hGRF analogues of small ring size. In our strategy the linear precursor

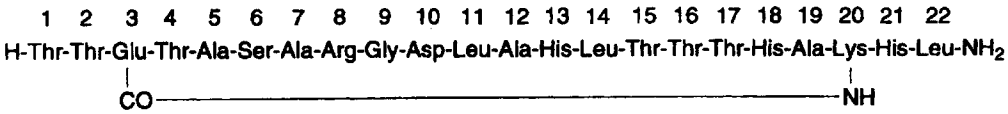

Figure 1. Primary structure of peptide **1**, reproducing residues 134–155 of VP1 from foot-and-mouth disease virus.

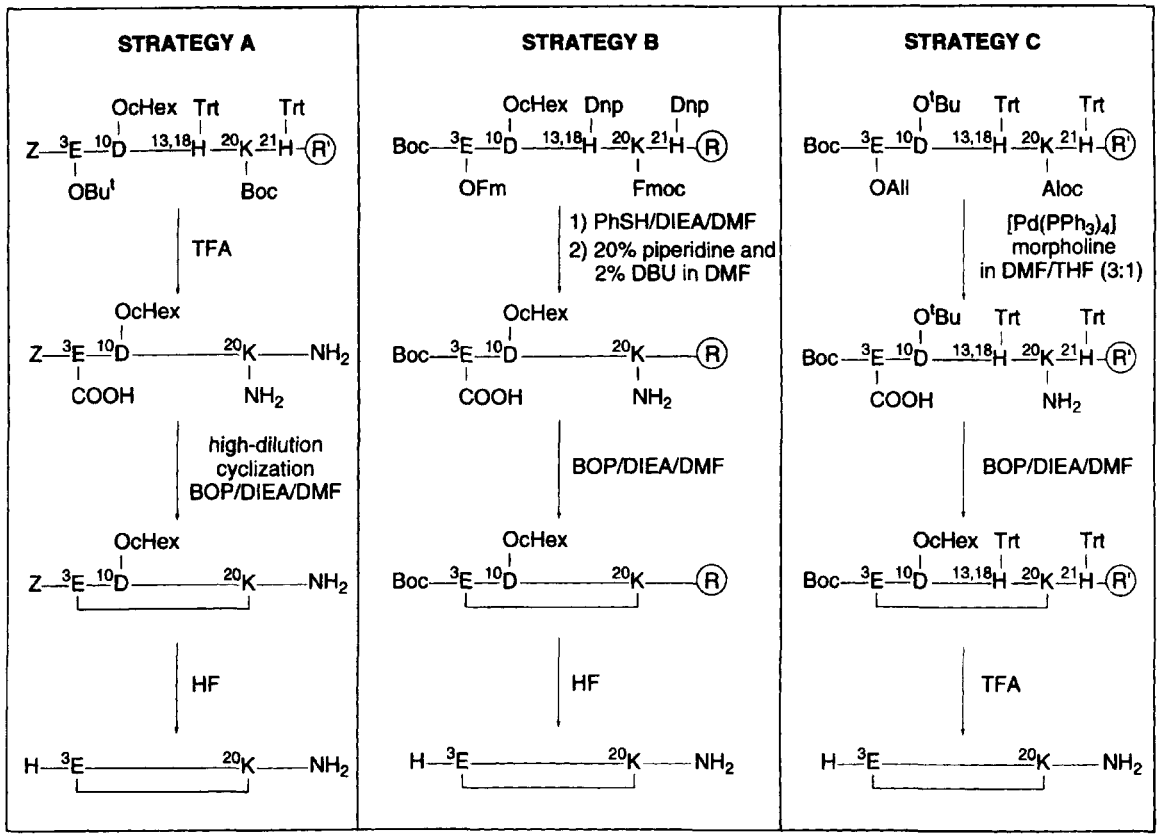

Figure 2 Synthetic approaches to peptide **1**.

was synthesized by conventional Boc/benzyl chemistry on a *p*-MBHA resin with low substitution, to avoid interchain side reactions. The sidechain functions of Glu^3 and Lys^{20} were protected with the OFm and Fmoc groups, respectively. These groups are fully orthogonal with the chemistry employed for chain assembly and can be selectively deprotected prior to solid-phase cyclization. Particular attention was given to the protection of the three His residues. We and others [28] have observed that the 2,4-dinitrophenyl (Dnp) protection is unstable to the conditions used for removal of the N^{ε}-Fmoc group (piperidine–DMF), reacting with the newly formed ε-NH_2 to give N-Dnp lysine. To avoid this side reaction, one possibility is first to remove the N^{im}-Dnp group by thiolysis, then to deprotect both the OFm and the Fmoc groups and carry out the cyclization in the presence of free His. The acylation of unprotected imidazoles by activated carboxyl groups is a described side reaction in peptide synthesis ([5], pp. 184–186). In this particular case, the resulting acylimidazoles could act as acylating agents that react with the Lys residue and contribute to the intramolecular cyclization reaction (assuming pseudo-dilution conditions). In the solid phase, however, accessibility of these acylating species to the amino group may be somewhat restricted; any unreacted acylimidazole will give rise to linear peptide upon acidolysis.

Another possibility, the use of a different protection for His residues, is not entirely satisfactory, since none of the alternatives is free from particular drawbacks. Thus, the N^{im}-tosyl group presents limited stability toward weak acids such as HOBt [29] while formaldehyde released during cleavage of the N^{π}-benzyloxymethyl group also causes various side reactions [30]. These considerations led us to choose the first alternative, i.e. removal of His(Dnp) protection prior to OFm and Fmoc deblocking and cyclization. The fully protected peptide resin was treated first with PhSH in order to deprotect the His residues and the with 20% piperidine and 2% DBU in DMF to remove the OFm and Fmoc groups. A little aliquot of partially deprotected resin was submitted to HF acidolysis to check the quality of the linear precursor (Figure 4A), which was correct by AAA and ES-MS. On-resin cyclization was carried out by treating the partially deprotected peptide resin with

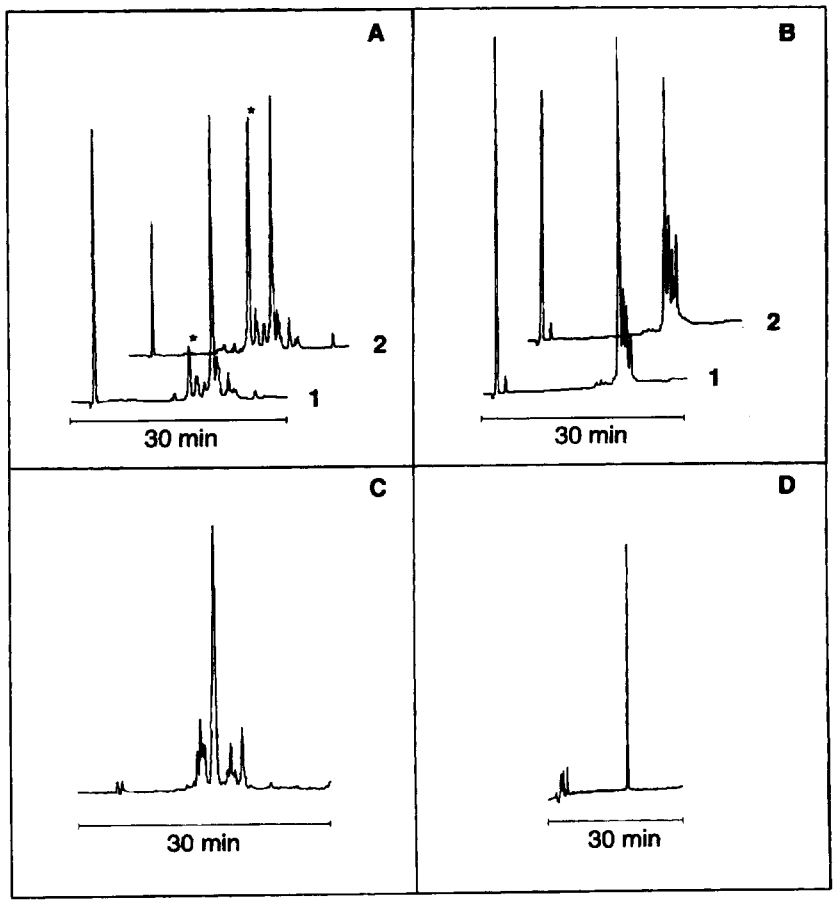

Figure 3. HPLC analysis of the synthesis and cyclization of peptide **1** by strategy A. Panel A: crude, partially protected [Z-N^{α}, Asp^{10}(OcHex)] linear peptide **1** after TFA cleavage with reagent B (trace 1) or reagent R (trace 2); the peak with an asterisk corresponds to the loss of the N^{α}-Z protection. Panel B: The previous product, purified by MPLC (not shown), cyclized with BOP (trace 1) or PyBOP (trace 2). Panel C: product from panel B (BOP cyclization) after total deprotection with HF. Panel D: cyclic peptide **1** after HPLC purification.

BOP/DIEA (15 and 30 equiv. added every 6 h, respectively) in DMF. The progress of the cyclization was monitored by the ninhydrin [31] test of peptide resin aliquots drawn at different reaction times, as well as by HPLC analysis of similar aliquots after HF cleavage. After 18 h, when no further progress of the reaction was observed, the peptide resin was treated with anhydrous HF to give a product (Figure 4B) with a major component that could be purified by MPLC to an HPLC-homogeneous product (Figure 4C) that coeluted with peptide **1** (prepared by strategy A) and had the expected mass by ES-MS. The presence of small amounts of linear peptide in the cyclization product (Figure 4B) could be attributed to N^{im}-acylation of some free His residues by the activated carboxyl group of the Glu residue [28]. The resulting acyl-imidazoles would revert to free carboxyl and imidazole groups upon acidolysis and yield the linear peptide. The broad peak absorbing at 340 nm contains residual Dnp-containing by-products that can be removed in the subsequent purification step.

Strategy C

This approach, also applied to the synthesis of small ring-size cyclic hGRF analogues [32], entirely avoids the presence of free His residues during the cyclization. The linear precursor was synthesized by Fmoc/^{t}Bu-type chemistry on low-substitution AM-PEG–PS resin. The sidechain functions of Glu^{3} and Lys^{20} were respectively protected with OAl and Aloc

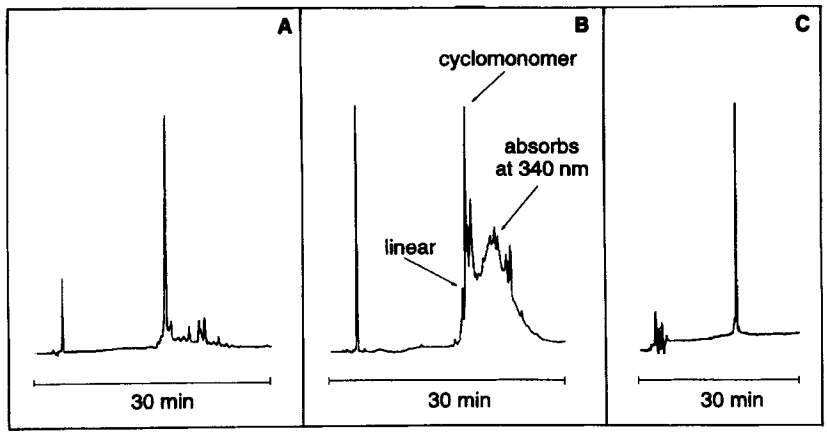

Figure 4 HPLC analysis of the synthesis and cyclization of peptide **1** by strategy B. Panel A shows the fully deprotected linear precursor cleaved from the resin. This intermediate, not strictly involved in strategy B, is shown to illustrate the quality of the synthetic process. Panel B: crude product after 18 h cyclization and HF cleavage. Panel C: final product **1** after MPLC purification.

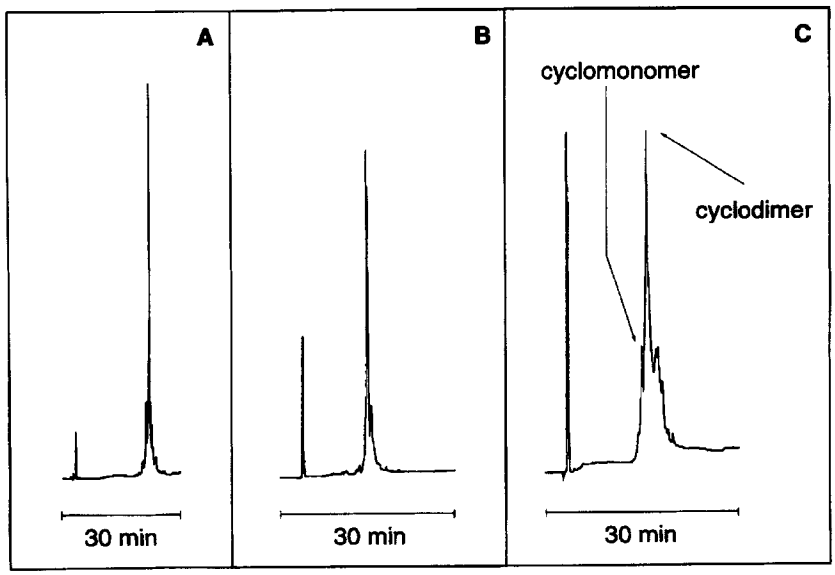

Figure 5. HPLC analysis of the synthesis and cyclization of peptide **1** by strategy C. Panel A shows the partially protected [Glu^3(OAl), Lys^{20}(Aloc)] linear precursor of peptide **1** after TFA cleavage. Panel B: fully deprotected linear peptide. The intermediates of both panels A and B, though not strictly involved in strategy C, serve to illustrate the quality of the synthetic process. Panel C: crude product after 18 h cyclization and TFA cleavage.

groups. Boc–Thr(^{t}Bu) was incorporated instead of Fmoc–Thr(^{t}Bu) as the N-terminal residue to avoid Fmoc deprotection by morpholine during allyl group removal. Figure 5A and B illustrates the quality of the resin-bound peptide after chain assembly and allyl deprotection with [$Pd(PPh_3)_4$]/morpholine in DMF/THF (3 : 1; overnight), respectively. On-resin cyclization was accomplished with BOP/DIEA (3 and 6 equiv. added every 6 h, respectively) in DMF. The reaction was monitored as in strategy B and stopped likewise after 18 h. Treatment of the peptide resin with TFA gave a material (Figure 5C), the main

component of which was identified by MS as the cyclodimer, while the cyclomonomer was the minor product. This somewhat unexpected result is clearly the result of intersite reaction (i.e. failure to achieve pseudo-dilution conditions), a situation that might be tentatively attributed to the higher flexibility of the PEG–PS resin (versus the more rigid *p*-MBHA used in strategy B).

Determination of Sidechain-to-Sidechain Bond Connectivity

In order to discriminate between the desired cyclic structure of peptide **1** and other possible cyclomonomeric arrangements not involving the Lys residue (*e.g.* lactone formation between Glu and Ser residues), the cyclic peptide prepared by strategy A was submitted to tryptic hydrolysis. As shown in Figure 6, participation of the Lys residue in the cyclic structure of **1** results in a single possible tryptic product with the same amino acid composition, whereas other cyclomonomeric arrangements involving neither Lys nor Arg residues will afford at least two peptide fragments. As shown in Figure 7, tryptic digestion of the purified final product from strategy A gave a major component by HPLC and HPCE (not shown) that did not coelute with the starting cyclic peptide and which has an amino acid composition identical to the parent peptide. ES-MS analysis of this peak gave a molecular peak 18 units higher than the cyclic peptide. This finding confirmed the correct structure of **1**.

General Comments

The present work demonstrates that large, fairly complex (e.g. rich in His residues) sidechain-to-sidechain peptide macrolactams can be approached by either solution or solid-phase cyclization methods. In the first instance (strategy A), we have used a minimal sidechain protection scheme (free Thr, Ser, Arg and His) which predictably improves solubility and purification of the linear precursor. Our results for this particular peptide indicate that cyclization yields in solution (strategy A) are consistently better than those on the solid phase (strategy B); however, the latter approach requires fewer purification steps, and therefore overall yields for either strategy end up being quite similar. The rather unexpected cyclodimer formation as the main outcome of strategy C reemphasizes the need to combine optimal protection strategies with polymer supports capable of affording adequate pseudo-dilution conditions required for large size peptide cyclization.

EXPERIMENTAL PART

Protected L-amino acids were from Advanced Chemtech (USA), Milligen (USA), Propeptide (F), Bachem (CH) or Novabiochem (CH). *p*-MBHA resin (0.81 mmol/g) and Fmoc–AM–AA–PEG–PS resin (0.2 mmol/g) were from Peptides International (USA) and Milligen, respectively. Other peptide synthesis reagents and solvents were of the best available commercial quality.

HPLC was performed on a Nucleosil C18 column (5 μm, 0.4 × 25 cm) eluted with 5–65% linear gradients of solvent B into solvent A [A: H_2O (0.045% TFA), B: MeCN (0.036% TFA)] at a flow rate of 1 ml/min. UV detection was at 220 nm. Purified peptides were characterized by amino acid analysis [6 M HCl, 110 °C, 24 h hydrolysis; Beckman 6300 analyser] and FAB- or ES-MS (VG Quattro, Fisons Instruments).

Tryptic digestion was performed as previously described [22]. The proteolysis product was analysed by HPLC on the above Nucleosil C18 column eluted with isocratic 1% B over 3 min followed by a linear 1–42% B gradient over 42 min. The main peak was collected, analysed for homogeneity by HPCE (fused silica 72 cm capillary; 50 cm to detector; 50 μm i.d.) at 210 V/cm and 30 °C in 20 mM citrate, pH 2.5 buffer and for identity by amino acid analysis and ES-MS; calculated molecular weight: 2331.7; found 2331.3.

Strategy A: Synthesis of Z–Thr-Thr(^{t}Bu)-Glu(O^{t}Bu)-Thr(^{t}Bu)-Ala-Ser(^{t}Bu)-Ala-Arg(Pmc)-Gly-Asp(OcHex)-Leu-Ala-His(Trt)-Leu-Thr(^{t}Bu)-Thr(^{t}Bu)-Thr(^{t}Bu)-His(Trt)-Ala-Lys(Boc)-His(Trt)-Leu-AM-Phe–PEG–PS resin

The synthesis was carried out in a Milligen 9050 Plus PepSynthesizer on a 0.2 mmol scale. Fmoc-amino acids were coupled using TBTU/HOBt/DIEA (1 : 1 : 2, × 4 equiv., 60 min) in DMF. Double couplings were made for the N-terminal section of the peptide, residues Thr^{1,2,4} and Glu^3. Fmoc removal was done with 20% piperidine in DMF (1 + 5 min, except for the first coupling, which was 1 + 10 min). Finally, the Z–Thr–OH residue was introduced manually using BOP/DIEA (5 and 10 equiv., respectively, 60 min) in DMF. Amino acid analysis of the peptide resin hydrolysate [12 M HCl/propionic acid (1 : 1), 2 h, 155 °C] gave Phe 1.0 (1, internal standard), Asp 1.0 (1), Thr 3.9 (6), Ser 0.6 (1), Glu 0.7 (1), Gly 0.9 (1), Ala 3.6 (4), Leu 2.9 (3), His 3.0 (3), Lys 1.0 (1), Arg 0.9 (1).

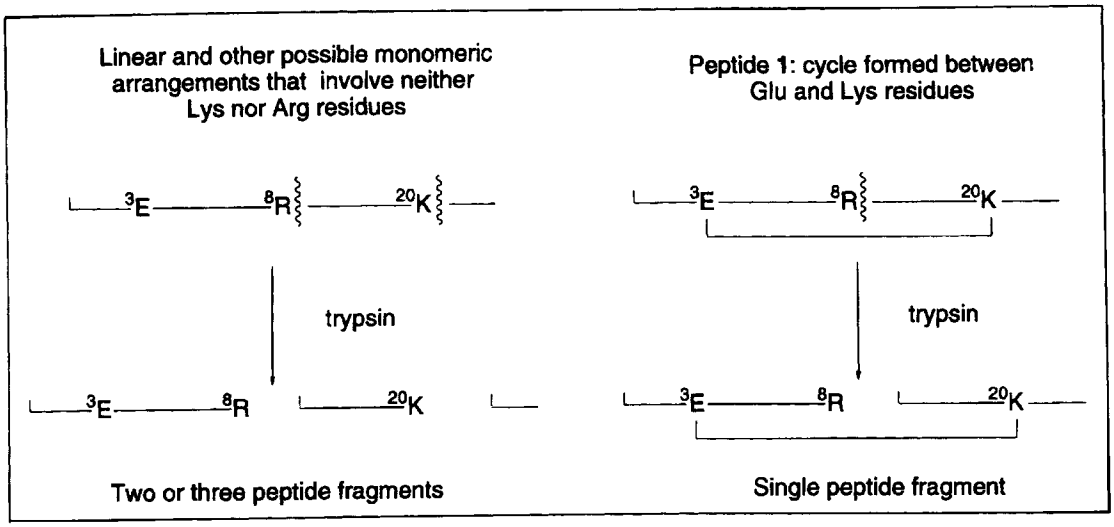

Figure 6. Predicted tryptic digestion patterns of linear and cyclic versions of peptide **1**

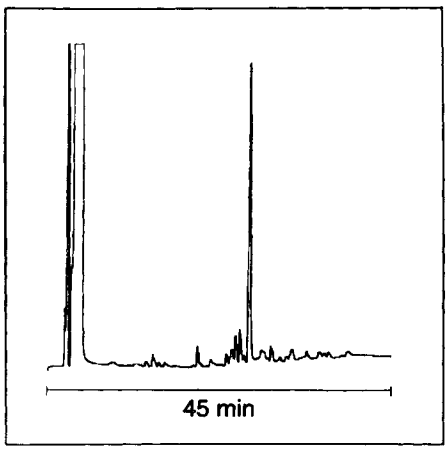

Figure 7. HPLC tryptic map of peptide **1**.

Cleavage and Partial Deprotection of Z-Thr(^{t}Bu)-Thr(^{t}Bu)-Glu(O^{t}Bu)-Thr(^{t}Bu)-Ala-Ser(^{t}Bu)-Ala-Arg(Pmc)-Gly-Asp(OcHex)-Leu-Ala-His(Trt)-Leu-Thr(^{t}Bu)-Thr(^{t}Bu)-Thr(^{t}Bu)-His(Trt)-Ala-Lys(Boc)-His(Trt)-Leu-AM-PEG-PS resin

Protected peptide resin (500 mg) was treated with freshly prepared TFA–anisole–H_2O (90:5:5 v/v, 5 ml) or reagent B (TFA–PhOH–HSi^{i}Pr$_3$–H_2O, 88:5:2:5 v/v, 5 ml) or reagent R (TFA–thioanisole–EDT–anisole, 90:5:3:2 v/v, 5 ml) at room temperature for 4 h. The resin was further washed with 5 ml of cleavage reagent. The combined filtrates were added to ice-cold Et_2O (*ca.* 40 ml) to precipitate the peptide material, which was separated by centrifugation. The pellet was washed with further cold Et_2O (4 × 40 ml) and redissolved in 0.1 M HOAc (10–20 ml). Yield (synthesis + acidolysis): 65%.

Purification and Cyclization of Z-Thr-Thr-Glu-Thr-Ala-Ser-Ala-Arg-Gly-Asp(OcHex)-Leu-Ala-His-Leu-Thr-Thr-Thr-His-Ala-Lys-His-Leu-NH_2

The above crude linear peptide was purified by MPLC (Michel-Miller C18 column, 2 × 30 cm) using a linear 20–25% gradient of MeCN in water (both solvents with 0.05% TFA). The usual batch size was 25 μmol (*ca.* 60 mg). The flow rate was *ca.* 170 ml/h and the eluate was monitored at 223 nm and collected into *ca.* 3 ml fractions which were analysed by HPLC and HPCE using the standard conditions described above. Fractions of satisfactory purity were pooled to give 8 μmol (*ca.* 20 mg, 32%) of material (> 90% by HPCE). FAB-MS: calculated molecular weight 2547.8; found 2547.6. This purified material was lyophilized five times from water and dried over P_2O_5 and NaOH to remove any residual HOAc, then diluted in DMF (stored over 4 Å molecular sieves and freed of amine contaminants by purging with N_2) to 50 μM concentration. DIEA was then added to 0.5% (v/v) concentration. The cyclization was started by the addition of BOP or PyBOP (20 equiv. every 6 h). The reaction was kept at room temperature under N_2 atmosphere and slow stirring for 18 h until cyclization was shown to be complete by HPLC; it was then quenched by addition of 0.5 M HCl in water to pH 3–4. The solvent was removed by evaporation, redissolved in a minimal amount of water and desalted on Sephadex G-15 (2 × 100 cm) in 0.1 M HOAc. All peptide fractions corresponding to the partially

protected cyclic amide peptide were pooled, lyophilized and fully deprotected in anhydrous HF/*p*-cresol (95:5 v/v, 5 ml 0°C for 20 min). After HF evaporation, the peptide residue was triturated with dry ether and DCM, dissolved in 0.1 M HOAc (14 ml) and purified by preparative HPLC on a Vydac C18 column (5 μm, 1 × 25 cm) eluted with a linear 14–17% gradient of solvent B into A (see above) over 45 min, at a flow rate of 3 ml/min. Fractions corresponding to cyclic amide peptide were pooled and lyophilized to give 1 μmol (*ca.* 2.5 mg, 13%) of material of satisfactory purity (97% by HPCE and HPLC). ES-MS: calculated molecular weight, 2313.7; found, 2313.9.

Strategy B: Synthesis of Boc–Thr(Bzl)-Thr(Bzl) Glu(OFm)-Thr(Bzl)-Ala-Ser-Ala-Arg(Tos)-Gly-Asp(OcHex)-Leu-Ala-His(Dnp)-Leu-Thr(Bzl)-Thr(Bzl)-Thr(Bzl)-His(Dnp)-Ala-Lys(Fmoc)-His(Trt)-Leu–*p*-MBHA resin

The C-terminal Leu[22] was coupled sub-stoichiometrically to *p*-MBHA resin by the DCC method to give a substitution of 0.2 mmol/g. The remaining free amino groups in the polymer were capped with Ac_2O and DIEA (5 mmol each) in DMF. The synthesis was continued in an Applied Biosystems 430A synthesizer at a 0.2 mmol scale using the standard (std1) files of the manufacturer, with minor modifications. The last four residues were coupled manually with BOP/DIEA (5 and 10 equiv., respectively; 60 min) in DMF. Manual Boc removal was done with neat TFA (1 + 10 min). AAA of a peptide resin hydrolysate gave Asp 1.1 (1), Thr 3.8 (6), Ser 0.4 (1), Glu 1.1 (1), Gly 1.1 (1), Ala 4.2 (4), Leu 3.2 (3), His 2.5 (3), Lys 0.9 (1), Arg 0.9 (1).

Solid-phase Cyclization of Boc–Thr(Bzl)-Thr(Bzl)-Glu (OFm)-Thr(Bzl)-Ala-Ser-Ala-Arg(Tos)-Gly-Asp(OcHex)-Leu-Ala-His(Dnp)-Leu-Thr(Bzl)-Thr(Bzl)-Thr(Bzl)-His(Dnp)-Ala-Lys(Fmoc)-His(Trt)-Leu–*p*-MBHA: Cleavage and Purification

Protected peptide resin (300–400 mg) was treated with PhSH/DIEA/DMF (3:3:4 v/v, 6 × 5 ml, 60 min) in DMF, then with 20% piperidine and 2% DBU in DMF (1 + 20 min). The cyclization on the resin was carried out with BOP/DIEA (20 and 60 equiv. every 6 h, respectively) in DMF at room temperature for 18 h and followed by N^{α}-Boc removal with neat TFA (1 + 10 min). Cleavage and total deprotection of the cyclic peptide resin was accomplished with anhydrous HF–*p*-cresol (9:1, v/v, 5 ml, 0 °C for 1 h). After HF evaporation, the residue was treated as above and the peptide was extracted into 0.1 M HOAc (10–15 ml). After lyophilization, the product was purified by MPLC on C18 silica using a linear gradient of 14–18% MeCN in water (both containing 0.05% TFA). Fractions corresponding to cyclomonomeric peptide were pooled to give 1.2 μmol (*ca.* 3 mg, 5%) of material of satisfactory purity (>95% by HPLC and HPCE).

Strategy C: Synthesis of Boc–Thr(^{t}Bu)-Thr(^{t}Bu)-Glu (OAll)-Thr(^{t}Bu)-Ala-Ser(^{t}Bu)-Ala-Arg(Pmc)-Gly-Asp(OcHex)-Leu-Ala-His(Trt)-Leu-Thr(^{t}Bu)-Thr(^{t}Bu)-Thr(^{t}Bu)-His(Trt)-Ala-Lys(Aloc)-His(Trt)-Leu–AM–*Nle*–PEG–PS resin

The synthesis was carried out in a Milligen 9050 Plus PepSynthesizer as in strategy A. The last four residues were coupled manually as in strategy B. Manual Fmoc removal was done with 20% piperidine and 2% DBU in DMF. Amino acid analysis of the peptide resin hydrolysate gave Asp 1.0 (1), Thr 3.4 (6), Ser 0.6 (1), Glu 08. (1), Gly 1.0 (1), Ala 3.9 (4), Leu 3.1 (3), His 3.1 (3), Lys 1.0 (1), Arg 1.1 (1).

Solid-phase Cyclization of Boc–Thr(^{t}Bu)-Thr(^{t}Bu)-Glu(OAll)-Thr(^{t}Bu)-Ala-Ser(^{t}Bu)-Ala-Arg(Pmc)-Gly-Asp(OcHex)-Leu-Ala-His(Trt)-Leu-Thr(^{t}Bu)-Thr(^{t}Bu)-Thr(^{t}Bu)-His(Trt)-Ala-Lys(Aloc)-His(Trt)-Leu–AM–*Nle*–PEG–PS resin. Cleavage and Purification

Protected peptide resin (300 mg, 33 mmol) was treated with [$Pd(PPh_3)_4$] (1.6 equiv.) and 10% morpholine in DMF–THF (3:1 v/v, 4 ml) under an Ar stream at room temperature overnight. The resin was then washed with 0.5% sodium diethyldithiocarbamate and 0.5% DIEA in DMF (3 × 5 ml, 3 min), DMF (3 × 5 ml) and finally DCM (3 × 5 ml). Cyclization on the solid support was carried out with BOP/DIEA (3 and 6 equiv. respectively, every 6 h) in DMF at room temperature for 18 h. The cyclic peptide resin was then cleaved and totally deprotected with reagent R as described in strategy A. The acidolysis product was purified in the same chromatographic system and conditions as above using linear gradients of 13–20% MeCN in water (+0.05% TFA). The main product was identified as the cyclodimeric peptide.

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